ACCUMULATION OF RECOMBINANT CELLOBIOHYDROLASE AND ENDOGLUCANASE IN THE LEAVES OF MATURE TRANSGENIC SUGAR CANE

Mark D. Harrison [a]*, Jason Geijskes [a], Heather D. Coleman [a¶], Kylie Shand [a], Mark Kinkema [a], Anthony Palupe [a¶], Rachael Hassall [a], Manuel Sainz [a†], Robyn Lloyd [a], Stacy Miles [b]‡ and James L. Dale [a]

[a] Syngenta Centre for Sugarcane Biofuels Development, Centre for Tropical Crops and Biocommodities, Queensland University of Technology, Brisbane, Queensland 4001, Australia; [b] Syngenta Biotechnology Inc, Research Triangle Park, 3054 East Cornwallis Road, NC 27709-2257, USA.

¶ Present address: Centre for Tropical Crops and Biocommodities, Queensland University of Technology, Brisbane, Queensland 4001, Australia

† Present address: Syngenta Proteção de Cultivos Ltda., Av Braz Olaia Acosta, 727 sala 1308, Jardim Califórnia, Ribeirão Preto - São Paulo, Brazil, CEP 14026-040

* To whom correspondence regarding the publication should be addressed: Syngenta Centre for Sugarcane Biofuels Development, Centre for Tropical Crops and Biocommodities, Queensland University of Technology, 2 George Street, Brisbane, Queensland 4001, Australia Tel.: 61-7-3138-9251; Fax: 61-7-3138-1534; E-mail: md.harrison@qut.edu.au

‡ To whom requests for materials should be addressed: Syngenta Biotechnology Inc, Research Triangle Park, 3054 East Cornwallis Road, NC 27709-2257, USA; Email: stacy.miles@syngenta.com

Author email addresses: md.harrison@qut.edu.au, jason.geijskes@syngenta.com, heather.coleman@qut.edu.au, kylie.shand@qut.edu.au, mark.kinkema@qut.edu.au, anthony.palupe@qut.edu.au, rachael.hassall@qut.edu.au, manuel.sainz@syngenta.com, robyn.lloyd@qut.edu.au, stacy.miles@syngenta.com, j.dale@qut.edu.au

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Summary

Reducing the cost of cellulolytic enzymes is a major strategic goal in making ethanol from lignocellulosic biomass a cost-competitive liquid transport fuel. Current production costs for these enzymes are high because they are produced by microbes. Expression of cellulolytic enzymes *in planta* is one way to substantially reduce production costs, but high-level accumulation of cellulolytic enzymes that can hydrolyse lignocellulosic substrates to fermentable sugars is required. Sugar cane fibre (bagasse) is the most promising lignocellulosic feedstock for conversion to ethanol in the tropics and sub-tropics. Cellulolytic enzyme production in sugar cane will have a substantial impact on the economics of lignocellulosic ethanol production from bagasse. Therefore, we generated transgenic sugar cane accumulating three different cellulolytic enzymes. The maize *PepC* promoter proved a useful alternative to maize *Ubi1* for driving expression of transgenes in sugar cane leaves. Different sub-cellular targeting signals were shown to have a significant impact on accumulation of fungal cellobiohydrolase I (CBH I) and cellobiohydrolase II (CBH II), and bacterial endoglucanase (EG) in leaves of mature sugar cane. The CBHs and EG accumulated to higher levels when fused to a vacuolar-sorting determinant compared to an ER-retention signal. EG, however, accumulated to the highest levels in sugar cane leaves when fused to a chloroplast-targeting signal. These results are the first demonstration of expression and accumulation of recombinant CBH I, CBH II and EG in sugar cane and represent a significant first step toward optimisation of cellulolytic enzyme expression in sugar cane for the economic production of lignocellulosic ethanol.

Introduction

Biofuels, including ethanol, are derived from renewable, biological feedstocks. The transition from petroleum to biomass feedstocks for production of liquid transport fuels offers the opportunity to improve global energy security, reduce greenhouse gas emissions, and enhance rural development (Fulton et al., 2004). The majority of fuel ethanol is produced by fermentation of sugars naturally present in sugar cane juice or molasses, or derived from the enzymatic hydrolysis of corn starch (Marris, 2006; Wheals et al., 1999). Sustainability benefits of biofuels are both feedstock and production technology-dependent; ethanol from corn starch provides modest gains in net energy production and reduction in greenhouse gas emissions whereas ethanol from sugar cane juice has a highly positive energy balance and large reduction in greenhouse gas emissions when substituted for an energy-equivalent amount of petroleum (Fulton et al., 2004; Macedo et al., 2008). Ethanol can also be produced from lignocellulosic feedstocks, including agricultural residues such as corn stover and sugar cane fibre (bagasse), forestry and municipal wastes, and dedicated energy crops. These second generation biofuel feedstocks constitute a huge, global carbohydrate resource, are typically lower cost than first generation feedstocks (starch and sugar), and are usually surplus to agricultural systems or may utilise marginal agricultural land. Conversion of lignocellulosic feedstocks into fermentable sugars for ethanol production is projected to have a highly positive energy balance and enable large reductions in greenhouse gas emissions (Fulton et al., 2004).

Sugar cane bagasse is an attractive lignocellulosic feedstock for second generation ethanol production (Moreira, 2006). The average annual dry biomass yield from commercial sugar cane varieties (39 t ha^{-1} bagasse and trash) (Waclawovsky et al., 2010) are significantly higher than those of other biomass crops such as Miscanthus (29.6 t ha^{-1}), switch grass (10.4 t ha^{-1}) and maize (17.6 t ha^{-1} grain plus stover) (Heaton et al., 2008). Sugar cane is produced in over 120 countries on an area of over 24 million hectares (FAOSTAT, 2008) and its cultivation generates the highest annual tonnage of all crops, approximately 1.74 billion tonnes (FAOSTAT, 2008). Further, bagasse is an existing residue of the sugar cane production process which is burnt to provide energy for the sugar cane factory and revenue from the sale of surplus electricity. With improvements in crop yield and/or process energy efficiency, large amounts of bagasse will be available for alternative uses, such as ethanol production, while ensuring that fibre is still available to provide heat and power for processing. As is the case in Brazil, sugar cane juice can be re-directed for ethanol production in dual-purpose mills depending on the relative prices of sugar and ethanol (Goldemberg and Guardabassi, 2010). Lignocellulosic material is essentially composed of plant cell walls that need to be broken down before they can be used as a feedstock for ethanol production.

The plant cell wall is a resilient, structurally heterogeneous barrier composed of complex polysaccharides, lignin and proteins that provides structural support and resistance to degradation, in addition to playing a key role in plant defence against pathogens (Huckelhoven, 2007). Plants synthesise two types of cell walls; a flexible, polysaccharide-rich, primary cell wall and a thicker, stronger secondary cell wall that comprises the majority of plant biomass (Vogel, 2008). The chemical composition of

plant cell walls varies; the major components are cellulose (35 - 50%), hemicellulose (20 - 35%) and lignin (10 - 25%) (Saha, 2003). Cellulose is a linear polymer of cellobiose, a glucose dimer. These individual cellulose chains are typically arranged parallel to each other with extensive hydrogen bonding between them; the result is a stable, crystalline structure resistant to enzymatic hydrolysis (OSullivan, 1997). Hemicellulose is composed of several pentose and hexose sugars arranged in a branched, non-crystalline structure (Spiridon and Popa, 2005). Lignin is an essentially random polymer of phenylpropylene sub-units (coumaryl, coniferyl and sinapyl alcohols) joined by ether and carbon-carbon linkages that is found covalently bound to hemicellulose in grasses (Boerjan et al., 2003). The overall structure of lignocellulosic material is very complex, with cellulose micro-fibrils embedded within a lignin-hemicellulose matrix. As a result of this structural complexity and the inherent stability of its constituents, lignocellulosic biomass requires significantly more energy to break down to fermentable sugars than starch.

Cellulose is enzymatically hydrolysed by highly specific cellulolytic enzymes (Beguin and Aubert, 1994) produced naturally by a wide range of bacteria and fungi (Sun and Cheng, 2002). The enzymes required for efficient and complete degradation of cellulose to glucose are derived from three functional classes; (i) endoglucanase (endo-1,4-β-D-glucanase, EG) which attack regions of low crystallinity (i.e. amorphous regions), creating free chain ends; (ii) exoglucanase or cellobiohydrolase (exo-1,4-β-D-glucanase, CBH) which cleave cellobiose units from either the reducing (CBH I) or non-reducing (CBH II) free chain-ends, and (iii) β-glucosidase which hydrolyses cellobiose to glucose (Coughlan and Ljungdahl, 1988). EG and CBH enzymes act synergistically to

effect the extensive hydrolysis of crystalline cellulose (Wood, 1969). In addition to these three classes of cellulolytic enzymes, most organisms that degrade plant cell walls also produce additional enzymes that hydrolyse lignin and hemicelluloses (Duff and Murray, 1996) to enhance access of cellulolytic enzymes to cellulose.

Expression of cellulolytic enzymes *in planta* provides an opportunity to significantly reduce saccharification costs in the production of lignocellulosic ethanol by minimising the amount of microbial cellulolytic enzymes required for hydrolysis. Bacterial, fungal and plant cellulases have been expressed *in planta*. The majority of these studies have focused on expression of EGs and, in particular, E1 (or its catalytic domain, E1cd) from the thermotolerant, cellulolytic bacteria *Acidothermus cellulolyticus* (12 reports, reviewed in Taylor et al. 2008). Recombinant E1 accumulation as high as 26% total soluble protein (TSP) in leaves has been reported (Zeigler et al., 2000), although yields of 0.1 - 5% TSP are more typical (Taylor et al., 2008). In contrast, there are few studies describing expression of CBHs *in planta.* Bacterial CBH II from *Thermomonospora fusca* expressed under the control of the constitutive Mac promoter (Comai et al., 1990) accumulated to 0.02% and 0.002% TSP in the leaves of tobacco and alfalfa, respectively (Ziegelhoffer et al., 1999). Yu et al. (2007) reported expression of the same recombinant CBH II in tobacco chloroplast and accumulation between 3 and 4% TSP. Expression of fungal CBH *in planta* has also been reported; *Trichoderma reesei* CBH I expressed under the control of the CaMV 35S promoter in tobacco leaves and callus accumulated to 0.11% and 0.082% TSP (Dai et al., 1999), respectively, while expression of T. *reesei* CBH I in maize embryo using the maize globulin promoter yielded cellulase at 5.2% TSP (Hood et al., 2007).

Production of cellulolytic enzymes in transgenic sugar cane brings together *in planta* expression with the best biomass crop for lignocellulosic ethanol in the tropics and sub-tropics. Transformation systems have been developed for sugar cane using both microprojectile bombardment (Bower and Birch, 1992) and *Agrobacterium tumefaciens* (Arencibia et al., 1998; Elliott et al., 1998; Enriquez-Obregon et al., 1998). The risk of transgene release from sugar cane is minimal because commercial cultivars are exclusively vegetatively propagated and sugar cane pollen has low viability (Moore, 1976; Moore and Nuss, 1987). In addition, most sugar cane cropping systems harvest prior to flowering to avoid the resulting drop in sugar content (Berding and Hurney, 2005). Critically, transport of biomass to a central facility for large scale processing is an existing part of all commercial sugar cane production systems and there is clear economic benefit in integrating production of ethanol from sugar cane bagasse into conventional sugar and ethanol production facilities (O'Hara, 2010).

Success in developing and deploying promoters for transgene expression in sugar cane has been relatively limited and only the constitutive maize polyubiquitin (Zm-*Ubi1*) promoter (Christensen et al., 1992) has been widely used in sugar cane (reviewed in Lakshmanan et al., 2005). Sugar cane cultivars are highly polyploid hybrids between *Saccharum officinarum* and *S. spontaneum* (Cuadrado et al., 2004; D'Hont et al., 1996) and genomic complexity has been proposed as the primary cause for the challenges in deploying functional promoters in transgenic sugar cane. Several sugar cane promoters have been isolated and shown to drive recombinant protein accumulation in callus and/or young plants but not in the expected pattern in mature plants (Wei et al., 2003; Yang et al., 2003). In addition, several heterologous promoters

drive strong expression in sugar cane callus but little or no expression in mature plants, with some limited evidence for gene silencing (Ingelbrecht et al., 1999; Mudge et al., 2009; Wei et al., 2003). Additional studies are required to understand the complexity of promoter functionality in sugar cane.

Sub-cellular targeting of recombinant cellulolytic enzymes is a key factor in optimising accumulation in transgenic plants. While there are numerous reports describing cellulolytic enzyme expression *in planta*, few describe transgenic plants expressing cellulolytic enzymes targeted to different sub-cellular compartments. *A. cellulolyticus* E1 accumulated to a higher level in potato leaves when targeted to the chloroplast, compared to the vacuole (Dai et al., 2000a). Further, this enzyme accumulated to a higher level when targeted to the apoplast, compared to expression with the native N-terminal signal (Dai et al., 2000a). Ziegelhoffer et al. (2001) expressed *A. cellulolyticus* E1 and E1cd in tobacco and demonstrated that the apoplastic space was superior to the chloroplast or cytosol for accumulation in leaves, a trend that was confirmed by Dai et al. (2005). In addition, this study demonstrated an ER-retention signal enhanced accumulation of *A. cellulolyticus* E1 compared to vacuolar-targeting (Dai et al., 2005). In contrast, there has been only one reported description of the effect of different targeting signals on CBH accumulation *in planta* (Miles, 2009). Fungal CBH I expressed in both tobacco or corn leaves accumulated to higher levels when vacuolar-targeted compared to a fusion with an ER-retention signal (Miles, 2009).

Here we report the ability of the maize phosphoenolpyruvate carboxylase (Zm-*PepC*) promoter to successfully drive transgene expression in transgenic sugar cane

leaves. We also describe the effect of sub-cellular targeting signals on the accumulation of three cellulolytic enzymes in the leaves of mature sugar cane. This is the first report of cellulolytic enzyme expression in sugar cane and the results described herein are an important first step in using *in planta* expression of cellulolytic enzymes to enhance the economics of lignocellulosic ethanol production from sugar cane biomass.

Results

The expression of the *E. coli uidA* (GUS) reporter gene and genes encoding cellulolytic enzymes in sugar cane in the present study were under the control of either the green-tissue specific phosphoenolpyruvate carboxylase (*Zm-PepC*) promoter (Matsuoka et al., 1994) or the Zm-*Ubi1* promoter, with different signal sequences at the 5' and/or 3' end of the coding sequence to confer sub-cellular targeting (Table 1). Zm-*PepC*-CBH I-CHL and Zm-*PepC*-CBH II-CHL constructs were not included in the present study because they did not drive accumulation of CBH I or CBH II in tobacco and maize events (S. Miles, *personal communication*). Embryogenic callus from sugar cane cultivar Q117 was transformed by microprojectile bombardment with linearised expression cassettes excised from the GUS and cellulolytic enzyme expression constructs. At least 50 events per cellulolytic enzyme expression construct were regenerated and each event was analysed for the presence of the transgene using primary TaqMan analysis. At least 20 transgenic events per construct (242 events in total) were grown to maturity for characterisation of CBH I, CBH II and EG accumulation.

The Zm-PepC promoter is functional in the mature leaves of transgenic sugar cane

To determine if the Zm-*PepC* promoter could be used for the production of recombinant protein in sugar cane leaves, GUS was expressed under the control of the Zm-*PepC* promoter and compared to expression from the Zm-*Ubi1* promoter (Figure

1). Both the Zm-*PepC* and Zm-*Ubi1* promoters were capable of driving GUS expression in the green leaves of mature transgenic sugar cane. The highest level of GUS accumulation from the Zm-*PepC* promoter (2168 ± 36 ng mg^{-1} protein) was approximately 9 fold higher than the highest level of GUS accumulation from Zm-*Ubi1* (240 ± 51 ng mg^{-1} protein). Further, three Zm-*PepC* events gave higher GUS accumulation than the best Zm-*Ubi1* event (Figure 1).

To further assess the utility of the Zm-*PepC* promoter in transgenic sugar cane, we determined the ability of the Zm-*PepC* and Zm-*Ubi1* promoters to drive accumulation of a cellulolytic enzyme (CBH I fused to an ER-retention signal, CBH I-ER) in the green and senescent leaves of mature sugar cane (Figure 2). Given the range of CBH I-ER activity in the independent transgenic events and that different numbers of events were analysed for each promoter construct (Zm-*Ubi1* = 20 events, Zm-*PepC* = 41 events), the enzyme activity data were presented in the form of an activity profile graph. In this representation of the data, the average enzyme activities measured for each event (Y-axis) were ranked from lowest to highest (on the X-axis), with the line with the highest activity assigned as 100%. CBH I activity in the green and senescent leaves of the highest expressing Zm-*PepC* CBH I-ER event were approximately half that measured in the equivalent leaves of the highest expressing Zm-*Ubi1* CBH I-ER event (Figure 2a, Table 2). However, average CBH I activity in the green and senescent leaves from the ten highest expressing Zm-*PepC* CBH I-ER events were higher than those measured in the green and senescent leaves of the ten highest expressing Zm-*Ubi1* CBH I-ER events (Table 2). Together, the GUS and CBH I-ER expression data demonstrate that the Zm-*PepC* promoter drives significant transgene expression and

accumulation in sugar cane leaves, and is a valuable alternative to Zm-*Ubi1* for transgene expression in sugar cane. Therefore, the Zm-*PepC* promoter was used to control expression of cellulolytic enzymes in sugar cane leaves in the remainder of the study.

The addition of a vacuolar-sorting determinant enhances accumulation of fungal CBH I and CBH II in green and senescent sugar cane leaves

To test the effect of protein-targeting signals on the accumulation of recombinant CBH, CBH I and CBH II were fused to a vacuolar sorting determinant (VSD) or an ER-retention signal. CBH I activity was measured in both the green and senescent leaves for the majority of events (Figures 3a and b). The highest CBH I activity measured in green leaves of sugar cane expressing CBH I as a fusion with a vacuolar-sorting determinant (CBH I-VSD) was approximately five times that measured in the event expressing the highest level of active CBH I-ER (Figure 3a, Table 2). The positive effect of fusion to a VSD was also observed in the senescent leaves (Figure 3b, Table 2). Further, the average CBH I activity in the green and senescent leaves of the ten events expressing the highest levels of active CBH I-VSD was substantially higher than those measured in the corresponding leaves of the ten events expressing highest levels of active CBH I-ER (Table 2). While the maximum CBH I activity in green and senescent leaves were similar for CBH I-VSD, for CBH I-ER the maximum level of activity in senescent leaves was approximately 2.5 fold higher than in green leaves (Table 2).

CBH II activity was also measured in both the green and senescent leaves of the majority of mature transgenic sugar cane events analysed (Figures 3c and d). Accumulation of active CBH II in green leaves of events expressing CBH II as a fusion with a vacuolar-sorting determinant (CBH II-VSD) was higher than in green leaves of events expressing CBH II as a fusion with an ER-retention signal (CBH II-ER) (Figure 3c, Table 2). The positive effect of fusion to a VSD was observed in both green (Figure 3c) and senescent leaves (Figure 3d) but was more pronounced in senescent leaves. The average CBH II activity in the green and senescent leaves of the ten highest expressing CBH II-VSD events were higher than those measured in the green and senescent leaves of the ten events with the highest levels of active CBH II-ER (Table 2). In contrast to the results obtained for CBH I, the maximum level of CBH II activity observed in this study in senescent leaves expressing CBH II-ER was lower than the maximum activity observed in green leaves (Figures 3c and d, Table 2).

The addition of a chloroplast-targeting signal enhances accumulation of a bacterial EG in sugar cane leaves

EG was detected by the quantitative enzyme-linked immunosorbent assay (qELISA) in samples from the green leaves of events transformed with the three EG expression cassettes (Table 1 and Figure 4a). The maximum level of EG accumulation in green leaves expressing EG fused to a chloroplast-targeting signal (EG-CHL, 468.4 ± 41.9 ng mg^{-1} protein) was approximately 1.6 fold higher than the maximum accumulation observed for EG fused to a vacuolar-sorting determinant (EG-VSD, 281.3 ± 30.1 ng mg^{-1}

protein) and approximately 2.2 fold higher than the maximum accumulation observed for EG fused to an ER-retention signal (EG-ER, 210.1 ± 9.3 ng mg^{-1} protein). The enhancement of accumulation by fusion to sub-cellular targeting signals was also reflected in the average accumulation of EG in the top ten events for each construct: EG-CHL, 223.8 ng mg^{-1} protein (range = 100.2 - 468.4 ng mg^{-1} protein); EG-VSD, 119.6 ng mg^{-1} protein (range = 58.7 - 281.3 ng mg^{-1} protein); EG-ER, 89.2 ng mg^{-1} protein (range = 54.1 - 210.1 ng mg^{-1} protein). To determine if the accumulated EG was active, samples from the green leaves of selected events were analysed for both EG abundance and EG activity. EG activity was detected in all samples and activity data was found to correlate well with enzyme abundance determined using qELISA (R^2 = 0.91) (Figure 4b). In contrast to CBH I and CBH II, EG was detected in the senescent leaves of a single EG-CHL event at 5.5 ± 1.4 ng mg^{-1} protein.

Characterisation of accumulated cellulolytic enzymes in green sugar cane leaves by western blot analysis

Protein extracts from the green leaves of the five mature sugar cane events accumulating the highest levels of CBH I, CBH II, and EG activity per construct were analysed by western blot. The results were consistent across the events analysed from each construct (data not shown) and the results for the highest accumulating line per construct are presented in Figure 5. No cross-reacting proteins were detected in protein extracts from untransformed sugar cane for any of the three recombinant proteins analysed. The majority of recombinant CBH I-VSD and CBH I-ER protein was

detected in a single band with a molecular mass of approximately 45 kDa (Figure 5a) for each protein. The apparent mass of these proteins were approximately 8 kDa lower than the predicted molecular masses calculated from the amino acid sequences of the mature proteins (Table 1). Fungal CBH I used as the positive control for the western blot was expressed in and purified from *Aspergillus*. Recombinant *T. reesei* CBH I expressed in *Aspergillus* has been reported to be highly glycosylated (Jeoh et al., 2008). Therefore, the mobility of the fungal-expressed CBH I was not used as an indicator of the mobility of the full length plant-expressed CBH I protein. In addition to the major band detected, two minor bands were detected for the CBH I-VSD protein at molecular masses of approximately 49 kDa and 40 kDa (Figure 5a). A small proportion (approximately 10%) of CBH I-ER was detected at a molecular mass of approximately 56 kDa when the western blot was over-exposed (data not shown), which is the predicted mass of the mature, recombinant protein (Table 1). No such band was detected in CBH I-VSD samples, despite the increased abundance of this protein relative to CBH I-ER.

CBH II-VSD and CBH II-ER were both detected in two bands with similar masses and equivalent intensity (Figure 5b). The apparent masses of these recombinant proteins (CBH II-VSD, 41 kDa and 44 kDa; CBH II-ER, 42 kDa and 46 kDa) were either approximately 4 kDa or 7 kDa lower than predicted based on the amino acid sequences of the mature proteins (Table 1). Purified, *Aspergillus*-expressed CBH II was used as a positive control for the western blot. As noted above for CBH I, the mobility of the fungal-expressed CBH II protein was not used as an indicator of the mobility of the full

length plant-expressed CBH II protein. No higher mass forms of either recombinant protein were detected when the western blot was over-exposed (data not shown).

EG-CHL accumulated with an apparent mass of 73 kDa, which was consistent with the predicted mass (Figure 5c). *E. coli*-expressed EG was used as a positive control for the western blot, and was presumably not glycosylated. Therefore, the mobility of this protein was an indicator of the mobility of full length EG. A minor band was detected at a molecular mass of approximately 66 kDa for EG-CHL when the western blot was over-exposed. EG-ER and EG-VSD accumulated with an apparent mass of 84 kDa, which was approximately 10 kDa larger than the predicted mass of both proteins (Figure 5c). A small proportion (less than 5%) of EG-ER was detected at the same molecular mass as EG-CHL (73 kDa) when the western blot was over-exposed (data not shown). Further, approximately 10% of EG-VSD accumulated at the same mass as EG-CHL (73 kDa), with a minor form detected with an apparent mass of 62 kDa.

Discussion

For lignocellulosic ethanol to become cost-competitive with fossil fuels, significant improvement is required in feedstock composition, storage and transport, biomass pre-treatment, enzymatic hydrolysis and fermentation. Expression of recombinant cellulolytic enzymes *in planta* has been identified as a potential means to substantially reduce the cost of the enzymes used to hydrolyse lignocellulosic biomass into fermentable sugars (Himmel et al., 2007; Sainz, 2009; Sticklen, 2006; Taylor et al., 2008). Therefore, we generated transgenic sugar cane expressing three different cellulolytic enzymes (CBH I, CBH II and EG) from two key classes (endoglucanase and exoglucanase) and assessed their accumulation in the leaves of mature sugar cane.

There are relatively few constitutive, tissue-specific or inducible promoters that have been validated for deployment in sugar cane compared to the selection available for other crops. The Zm-*PepC* promoter was used in this study to drive transgene expression in sugar cane. The GUS reporter protein was shown to accumulate to a higher level in the green leaves of mature sugar cane when expressed under the control of the Zm-*PepC* promoter, compared to expression under the control of the Zm-*Ubi1* promoter. Further, the Zm-*PepC* promoter drove accumulation of CBH I in both green and senescent leaves of mature sugar cane. Our results are consistent with those previously reported for maize (Miles, 2009), where the Zm-*PepC* promoter drove recombinant CBH I-ER, CBH I-VSD and CBH II-VSD accumulation in both the green and senescent leaves of maize. Therefore, we have demonstrated the utility of the Zm-

PepC for transgene expression in the leaves of transgenic sugar cane and expanded the currently limited range of promoters available for use in this important crop.

The accumulation of active EG, CBH I and CBH II in transgenic sugar cane events generated in the present study did not induce an obvious altered phenotype. Several studies have described negative phenotypes ascribed to recombinant cellulolytic enzyme accumulation *in planta*. For example, Abdeev et al. (2003) observed an alteration in tobacco leaf shape and rugosity with apoplastic accumulation of thermostable *Clostridium thermocellum* CelE EG catalytic domain. Further, a significant reduction in the growth rate of transplastomic tobacco accumulating *A. cellulolyticus* E1cd (or the 10nE1cd variant) to approximately 12% TSP in the leaves was observed by Ziegelhoffer et al. (2009). In contrast, Ziegler et al. (2000) described high level (approximately 26% TSP) accumulation of apoplast-targeted *A. cellulolyticus* E1cd in the leaves of *Arabidopsis thaliana*, underscoring the importance of targeting to achieve accumulation of cellulolytic enzymes without adverse agronomic effects.

This study is the first to describe relative accumulation of cellulolytic enzymes targeted to different sub-cellular compartments in sugar cane. Human cytokine granulocyte macrophage colony stimulating factor accumulated to a higher level in leaves of transgenic sugar cane when fused to an ER-retention signal (HDEL), compared to a recombinant protein that was predicted to be exported to the apoplastic space (Wang et al., 2005). Avidin accumulated to its highest levels in sugar cane leaves, stem (internode 4) and roots when targeted to the δ-type vacuole, compared to the lytic vacuole, ER, apoplast or cytosol (Jackson et al., 2010). Our results demonstrate that translational fusion to a VSD enhanced the accumulation of CBH I

and CBH II in green sugar cane leaves when compared to fusion with an ER-retention signal. A range of vacuole types have been identified in the leaves of sugar cane (Rae et al., 2009); bulliform cells contain large acidic and proteolytic vacuoles, while vacuoles in the epidermal and bundle sheath cells also have strong proteolytic activity. The VSD used in the present study (DELKAEAK) has been demonstrated to target recombinant protein to the lytic vacuole during transient expression in tobacco protoplasts (Cervelli et al., 2004). Previous studies have also demonstrated accumulation of recombinant cellulolytic enzymes in transgenic events when fused to a VSD (Dai et al., 2000a; Dai et al., 2005) and, further, our results are consistent previous observations for fungal CBH I expression in both tobacco and maize leaves (Miles, 2009).

The majority of CBHI and CBH II expressed in the leaves of transgenic sugar cane accumulated with apparent molecular masses lower than was suggested by the amino acid sequences of the mature proteins. Given that these enzymes have retained their catalytic activity on surrogate substrates, it is likely that the reduction in apparent masses resulted from the loss of the CBD from both CBH I and CBH II. Further biochemical analyses will be needed to confirm the integrity of the CBH I and CBH II proteins expressed in sugar cane. In contrast to our results, Miles et al. (2009) demonstrated that recombinant CBH I-ER and CBH I-VSD expressed in maize leaves both accumulated with apparent masses either consistent with, or slightly greater than, the predicted masses of their mature proteins. Differences in the apparent mass of the same cellulase accumulated in different plants is not without precedent; recombinant bacterial CBH II (with an N-terminal CBD) expressed in the cytosol of

potato and tobacco leaves co-migrates with purified protein derived from the source bacteria (Ziegelhoffer et al., 1999). However, in the same study, it was demonstrated that approximately 50% of bacterial CBH II accumulated with mobility consistent with the predicted mass of the enzymes catalytic domain when expressed in the cytosol of alfalfa leaves. Our results, therefore, support the proposition that the integrity of a particular cellulolytic enzyme in one crop is not automatically a predictor of integrity in another.

Transgenic sugar cane events accumulated significantly higher levels of EG when targeted to the chloroplast, compared to ER or vacuolar-targeting. This was not entirely unexpected, given the prokaryotic origin of the plastid (Gould et al., 2008) and that the EG expressed was of bacterial origin. EG-CHL appeared as a single band on a western blot consistent with the predicted molecular mass of the mature protein. Further, the mobility of EG-CHL expressed in sugar cane was indistinguishable from that of control EG expressed in *E. coli* without any targeting signals, suggesting that the chloroplast-transit peptide was cleaved correctly and efficiently in sugar cane. Taken together, this evidence supports our hypothesis that the *Cyanophora paradoxa* FNR chloroplast-transit peptide functions in sugar cane to target recombinant protein to the chloroplast. However, further analysis will be required to determine if EG-CHL is being correctly targeted to the chloroplast in the transgenic sugar cane events described herein. In contrast to EG-CHL, the majority of EG-ER and EG-VSD appear to have a higher apparent molecular mass than either the control protein or EG-CHL. Given that N-linked glycosylation is initiated in the ER (Schachter, 1991), we would expect that any proteins fused to the γ-zein signal peptide would be glycosylated,

should they possess appropriate peptide sequences to signal the addition of N-linked sugars. The EG chosen for this study does indeed contain putative sites for the addition of N-linked sugars (data not shown). We propose, therefore, that the increase in apparent molecular mass of EG-ER and EG-VSD resulted from glycosylation during accumulation in sugar cane leaves. However, further analysis will be required to confirm the glycosylation of recombinant EG protein and to determine the effect, if any, of glycosylation on enzyme activity.

That the recombinant EG in transgenic sugar cane leaves does not appear to be significantly truncated is likely attributed to the fact that the EG utilised in this study is mono-domain and lacking a CBD. Contrast this with studies describing accumulation of *A. cellulolyticus* E1 (Dai et al., 2000a; Dai et al., 2000b; Dai et al., 2005; Sun et al., 2007; Ziegelhoffer et al., 2001) and *Thermobifida fusca* Cel6A (Ziegelhoffer et al., 1999) EGs in potato, tobacco, and duckweed, where a significant proportion of the recombinant enzymes were shown to be truncated. It is worth noting that, in these cases at least, truncation was not dependent upon the particular sub-cellular compartment to which the enzyme was targeted (Dai et al., 2000a; Dai et al., 2000b; Dai et al., 2005; Sun et al., 2007; Ziegelhoffer et al., 1999; Ziegelhoffer et al., 2001). Therefore, given that only a small proportion (approximately 1%) of the bacterial EG in the present study was truncated, and the diversity of targeting but consistency of truncation in the studies described above, it is possible that expression of bacterial EG *in planta* will result in the loss of the CBD if one is present in the mature protein sequence.

This study has demonstrated that CBH I and CBH II accumulate in senescent leaves of transgenic sugar cane when fused to either a VSD or ER-retention signal. The

CBH I-ER, CBH I-VSD, CBH II-ER and CBH II-VSD proteins expressed in this study have previously been shown to accumulate to higher levels in senescent transgenic corn leaves compared to green leaves (Miles, 2009). In contrast, the bacterial EG utilised in this study was only detected in the senescent leaves of one sugar cane event from the 92 events generated using three EG-expression cassettes and analysed at maturity. This was consistent with what was observed in transplastomic tobacco expressing *T. fusca* Cel6A (EG), where recombinant protein levels went from being easily detected in young leaves (approximately 0.25% total extracted protein) to being undetectable in senescent leaves by western blot (Yu et al., 2007). In contrast however, recombinant *A. cellulolyticus* E1 and E1cd accumulated in the senescing leaves of transgenic tobacco irrespective of targeting to the cytosol, chloroplast or apoplast (Ziegelhoffer et al., 2001). Further, active recombinant *A. cellulolyticus* E1 accumulated in the lower leaves of transgenic tobacco when targeted to the chloroplast (Dai et al., 2000b). It is difficult to establish a clear trend with respect to EG accumulation in senescent leaves of transgenic plants given the inconsistency between the specific enzymes expressed (all have different thermodynamic properties), and further work will be required to determine if the apparent reduction in EG abundance between young and old leaves in the present study was due to a particular sensitivity of this protein to the endo- and exo-peptidases released during leaf senescence (Lim et al., 2007).

We have demonstrated for the first time that the Zm-*PepC* promoter drives recombinant protein accumulation in the leaves of transgenic sugar cane. Further, we provide the first evidence for the accumulation of active, recombinant cellulolytic enzymes in transgenic sugar cane leaves. At high levels, accumulation of cellulolytic

enzymes in sugar cane could reduce or eliminate the need for the addition of commercially produced microbial enzymes for conversion of sugar cane bagasse to fermentable sugars. As such, this study represents an important first step towards the goal of producing ethanol from sugar cane bagasse at a price cost-competitive with fossil-derived, liquid transport fuels.

Experimental Procedures

Gene constructs

The Zm-*Ubi1*-*npt*II selection cassette, containing the maize *Ubi1* promoter (Christensen et al., 1992), was generated by Braithwaite et al. (2004). The *uidA* (GUS) coding region used in this study consisted of a 232 bp exon 1, 84 bp synthetic intron, and 1580 bp exon 2. The GUS coding region was amplified from p35S-GS (provided by Farmacule Bioindustries Ltd) by polymerase chain reaction (PCR) using primer 1 (5' – CCCGGGATCCTAAACCATGGTCCGTCCTGTAGAAACCC - 3') and primer 2 (5' - TCATTGTTTGCCTCCCTGCTG - 3') and KAPA HiFi DNA polymerase (Kapa Biosystems). The resulting PCR product was cloned into pGEM-T (Promega) and sequence verified. The GUS coding region was excised from pGEM-T using *Sma*I and *Not*I, treated with T4 DNA polymerase (Promega) to generate blunt ends and then ligated into the *Sma*I site of a pBluescript vector containing the Zm-*Ubi1* promoter and *nos* terminator (Zm-*Ubi1*-*nos*/pBS) to generate Zm-*Ubi1*-GUS. To construct Zm-*PepC*-GUS, the GUS coding region (amplified as described above) was cloned into pGEM-T and a clone containing the GUS coding region in the correct orientation was sequence verified to generate GUS/pGEM-T. The *nos* terminator was PCR amplified using primer 3 (5' – CCGCGGATCGTTCAAACATTTGGCAATA -3') and primer 4 (5' – GCATGCGATCTAGTAACATAGATGACA – 3'), and cloned into GUS/pGEM-T using *Sac*II and *Sph*I to generate GUS-*nos*/pGEM-T. The Zm-*PepC* promoter (Matsuoka et al., 1994)

and intron were excised from Zm-*PepC*-CBH I-ER using *Bam*HI and *Pst*I and sub-cloned into the corresponding restriction sites in GUS-*nos*/pGEM-T to generate Zm-*PepC*-GUS.

Cellulolytic enzymes utilized in this study were proprietary biomolecules obtained through a collaboration with Verenium Corporation (Stege et al., 2008); CBH I (Glycosyl hydrolase (GH) family 7) and CBH II (GH family 6) enzymes were derived from fungal isolates and are closely related to analogous enzymes found in *Penicillium* and *Trichoderma* sp., respectively. A mesophilic, bacterial EG (GH family 5) derived from environmental samples was also utilised. Cellulolytic enzyme expression vectors were designed in Vector NTI 9.0 using monocot optimised protein coding sequences. The amino acid sequences of the recombinants are presented in Supplementary Figure 1. Additional DNA sequences were added to the 5' and 3' end of each CBH I, CBH II, and EG coding regions for cloning and differential targeting to sub-cellular compartments. These sequences included a *Bam*HI cloning site, Kozak sequence, and N-terminal maize γ-zein signal sequence (MRVLLVALALLALAASATS) at the 5' end. VSD (DELKAEAK) or ER-retention (SEKEDEL) signals, and a *Sac*I cloning site were added at the 3' end. Silent mutations were introduced to remove any restriction sites which interfered with cloning strategies. Genes were synthesized by GENEART (Germany). The DNA sequences of the cellulase genes used in this study have been deposited in GenBank/EMBL and the accession numbers are indicated in Table 1.

Production of embryogenic sugar cane

Plant material from sugar cane variety Q117 was kindly provided by BSES Limited (Northern Experiment Station, Gordonvale, Qld, Australia). Transverse sections of immature sugar cane leaf whorls were prepared as described by Bower and Birch (1992). Sections were placed on embryogenic media (EM-D3; MS salts and MS vitamins (Murashige and Skoog, 1962), 20 g L^{-1} sucrose, 0.5 g L^{-1} casein hydrolysate, 10 % (v/v) young coconut juice, 8 g L^{-1} agar, 3 mg L^{-1} 2,4-Dichlorophenoxyacetic acid, pH 5.7) in deep Petri dishes (25 mm x 90 mm) sealed with micropore surgical tape (3M). Cultures were incubated at 25 °C in the dark, sub-cultured onto fresh media every 12 - 14 days and used for transformation six weeks after initiation.

Plant transformation and event selection

Transgene expression cassettes (promoter - gene - terminator) were excised from the vectors listed in Table 1 using restriction endonucleases, separated on a 0.8% (w/v) agarose gel and purified from excised gel slices using the QIAquick gel extraction kit (QIAGEN). DNA was eluted in 10 mM Tris pH 8.0, the concentration was adjusted to 100 ng μL^{-1} and used for microprojectile coating. One micron gold microprojectiles (Bio-Rad) were prepared as described by Dugdale et al. (1998), using 100 ng of GUS or cellulolytic enzyme expression cassettes and 100 ng of selection gene (Zm-*Ubi1-npt*II) cassette, and delivered using a high pressure helium (He) particle in-flow device (10 ms pulse of He at 1500 kPa) to accelerate micoprojectiles. A volume of 1 cm^3 of callus (approximately 30 calli) was used for each bombardment. After bombardment, callus was distributed into discreet clumps on selection media (EM-D3 media supplemented

with 45 mg L^{-1} Geneticin) and cultured for 4 weeks. Viable callus was transferred to regeneration media (MS salts, MS vitamins, 20 g L^{-1} sucrose, 8 g L^{-1} agar, 45 mg L^{-1} Geneticin, pH 5.7) and cultured for 6 weeks, ensuring callus clumps were separated at all stages. A single event was recovered from each callus clump and moved to rooting/elongation media (MS salts, MS vitamins, 30 g L^{-1} sucrose, 8 g L^{-1} agar, 45 mg L^{-1} Geneticin, pH 5.7).

Events were regenerated and transferred to soil after approximately 4 weeks on rooting/elongation media and acclimatised in the Queensland Crop Development Facility glasshouse. The glasshouse received ambient light and the temperature was set at 28 °C with evaporative cooling (maximum temperature was 32 °C). Three months post-transfer to soil, four leaf discs were taken from the first unfurled leaf of each event. DNA was extracted from the tissue and analysed for the presence of the transgene using TaqMan real-time PCR analysis following the methods described in Ingham et al. (2001). Non-transgenic Q117 regenerated from the same batch of callus used to generate the transgenic events were sampled and extracted at the same time as negative controls.

Leaf sampling for enzyme analysis

Samples were collected by hole-punch (approximately 7 mm diameter) from the first, fully unfurled leaf and last senescent leaf of each event at approximately mid-leaf length twelve months post-transfer to soil. Mid-rib sampling was avoided. Three samples of five leaf disks were collected per event and freeze-dried prior to protein

extraction. Non-transgenic Q117 regenerated from the same batch of callus used to generate the transgenic events were sampled and extracted at the same time as negative controls.

Cellulolytic enzyme activity assays

Sugar cane leaf proteins were extracted into 100 mM sodium acetate buffer pH 4.75 containing 0.02% (w/v) NaN_3, 0.02% (v/v) Tween® 20 and protease inhibitors (Complete, Roche) for analysis of CBH I, CBH II and EG enzyme activity. For analysis of CBH I activity, extraction buffer was supplemented with 27.2 mg (dry weight equivalent) of acid-washed Dowex 1X2-400 (Sigma) prepared as described by Loomis et al (Loomis et al., 1979). Protein concentration was measured using the BCA assay (Thermo Scientific, USA) for CBH I and CBH II activity analyses or the Bradford Assay (Bio-Rad Protein Assay) for EG activity analyses, relative to a BSA standard curve. Samples from the same event were assayed in adjacent wells for protein content and enzyme activity. All enzyme activity values reported were the mean of the data obtained for three samples per event and were normalised to the concentration of protein extracted from sugar cane leaves at pH 4.75.

CBH I activity was measured by monitoring the ability of protein extract to release fluorescent 4-Methylumbelliferone (4-Mu) from 4-Methylumbelliferyl-β-D-lactopyranoside (MUL) (Dai et al., 2005; van Tilbeurgh et al., 1982; van Tilbeurgh et al., 1988) after 20 minutes at 40 °C and pH 4.75, relative to a 4-Mu standard curve prepared in leaf extract from non-transgenic Q117. Fluorescence was measured using

an LS50B Luminescence Spectrometer (Perkin Elmer). CBH I activity was reported as nmol 4-Mu min^{-1} mg^{-1} protein.

CBH II activity was measured by monitoring the ability of protein extract to release reducing sugars from 1% (w/v) phosphoric acid swollen cellulose (PASC) at 40 °C and pH 4.75, relative to a glucose standard curve. PASC was prepared from Avicel PH-101 (Fluka) using the method described by Wood (1988). The amount of reducing sugars released after six hours was measured using the Biuret reaction (Johnston et al., 1998) on an AD200C microplate reader (Beckman Coulter). CBH II activity was reported as nmol reducing sugars min^{-1} mg^{-1} protein.

EG activity was measured by monitoring the ability of protein extract to release glucose from 0.5% (w/v) carboxymethyl cellulose (CMC-4M, Megazyme) at 40 °C and pH 4.75 in the presence of β-glucosidase (GOPOD Kit, Megazyme), relative to a glucose standard curve (Mandels et al., 1976). The amount of glucose released after two hours was measured using glucose oxidase/peroxidise chemistry (Megazyme) on a microplate reader (AD200C, Beckman Coulter). EG activity was reported as nmol glucose min^{-1} mg^{-1} protein.

GUS and EG quantitation by qELISA

For GUS quantitation, high-binding 96-well plates (Maxisorp, Nunc) were coated at 4 °C overnight with 2 μg mL^{-1} rabbit anti-GUS IgG (Sigma G5545) in binding buffer (25 mM borate, pH 8.5 containing 75 mM NaCl, 100 μl per well). Plates were washed three times with wash buffer (10 mM Tris, pH 8.0 containing 0.05% (v/v) Tween® 20 and

0.2% (w/v) NaN_3). Samples or standards (GUS Type VII-A, Sigma G7646) were added to the plate (100 μL per well), incubated for one hour at room temperature (RT) with shaking, and the plate was washed five times with wash buffer. Horseradish peroxidase conjugated rabbit anti-GUS IgG (2 μg mL^{-1}, 100 μL per well, Invitrogen) was then added to the plate, incubated for one hour at RT with shaking, and the plate was washed five times with wash buffer. Substrate (tetramethylbenzidine, Sigma) was added (100 μL per well) and developed for 30 minutes at RT. The reaction was stopped by the addition of 0.1 N HCl (100 μL per well). The absorbance was measured at 450 nm, with absorbance at 620 nm as a reference, using a microplate reader (Tecan Sunrise, Research Triangle Park, NC). The standard curve used a four-parameter curve fit to plot the concentration of GUS against absorbance at 450 nm. The range of detection for GUS was between ~7 and 320 ng mL^{-1} of sample extract using this method. Non-transgenic Q117 were analysed as negative controls. For the quantification of EG, the same method was utilised with the following alterations: plates were coated with 1 μg mL^{-1} rabbit anti-EG IgG, the antibody used for detection was 1 μg mL^{-1} alkaline phosphatase conjugated rabbit anti-EG IgG, the substrate used was p-nitrophenyl phosphate (Sigma), no HCl was added to stop the reaction and the absorbance was measured at 405 nm with absorbance at 492 nm as a reference. The minimum limit of detection for EG was one ng mg^{-1} protein.

Western blot analysis

Protein extracts for western blotting were prepared as described for cellulolytic enzyme activity assays and aliquots (10 μg) were resolved by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using standard conditions (Laemmli, 1970). Protein size standards (Novex® Sharp Pre-Stained Protein Standards, Invitrogen) and western size standards (MagicMark™ XP Western Standard, Invitrogen) were included on each gel. Resolved proteins were transferred to positively-charged nylon membranes (Roche) using a Mini Trans-Blot® Cell (Bio-Rad) and the membranes were blocked with 5% skim milk powder in TBST (20 mM Tris pH 7.5, 150 mM NaCl, 0.1% (v/v) Tween® 20). The membranes were probed with rabbit anti-CBH I, rabbit anti-CBH II and rabbit anti-EG primary antibodies (400 ng mL^{-1}), and the secondary antibody for all analyses was horseradish peroxidise (HRP)-conjugated goat anti-rabbit antibody (80 ng mL^{-1}, Thermo Scientific). Recombinant cellulolytic enzymes were visualised using HRP substrate (Super Signal West Femto Maximum Sensitivity Substrate, Thermo Scientific) at a 10% dilution in ultrapure water. Negative control extracts were generated from non-transgenic Q117. Denatured samples of recombinant CBH I, CBH II and EG were provided by Syngenta following expression and purification from either *Aspergillus niger* (CBH I and CBH II) or *E. coli* (EG) as positive controls.

In silico analysis of protein sequences

The amino acid sequences of the recombinant proteins were analysed using SignalP 3.0 (Emanuelsson et al., 2007) to predict the site of cleavage of ER transit peptides and

InterProScan (Zdobnov and Apweiler, 2001) to predict the presence or absence of cellulose-binding domains.

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Supporting Information

Figure S1 The amino acid sequences of the CBH I, CBH II and EG proteins expressed from the constructs used in this study. (a) Zm-*Ubi1*-CBH I-ER. (b) Zm-*PepC*-CBH I-ER. (c) Zm-*PepC*-CBH I-VSD. (d) Zm-*PepC*-CBH II-ER. (e) Zm-*PepC*-CBH II-VSD. (f) Zm-*PepC*-EG-ER. (g) Zm-*PepC*-EG-CHL. (h). Zm-*PepC*-EG-VSD. N-terminal signal peptides are underlined. C-terminal signals are double-underlined.

(a)

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(b)

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(c)

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SGDPKTVESQSGSSYVTFSDIRVGPFNSTFSGGSSTGGSSTTTASGTTTTKASSTSTSSTSTGTGVAAHWGQC
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(d)

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(e)

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(f)

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(g)

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Q

(h)

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ALLNGMMSGTGGTVPTPPTPPATPTPPTTITGNLGTYQFGTQEDGVSPNYTQAVWELSGTNLTTAKTTGA
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QPSLRIIIAYYNGGNVNDLGIVSANLTQDELKAEAK

Tables

Table 1 Vector components used for sugar cane transformation

Vector	GenBank Number	Promoter	N-terminal sequence	Transgene	Terminator	C-terminal sequence	Target	Predicted mass (kDa)
Zm-Ubi1-NptII	Submitted	Zm-*Ubi1* [1]	None	*nptII* [2]	tNOS	None	-	-
Zm-PepC-GUS	Submitted	Zm-*PepC* [3]	None	*uidA* [4]	tNOS	None	-	-
Zm-Ubi1-GUS	Submitted	Zm-*Ubi1*	None	*uidA*	tNOS	None	-	-
Zm-Ubi1-CBH I-ER	Submitted	Zm-*Ubi1*	γ-zein [5]	CBH I	tNOS	SEKDEL	ER	53.2
Zm-PepC-CBH I-ER	Submitted	Zm-*PepC*	γ-zein	CBH I	t*PepC*	SEKDEL	ER	53.2
Zm-PepC-CBH I-VSD	Submitted	Zm-*PepC*	γ-zein	CBH I	t*PepC*	DELKAEAK [6]	Vacuole	53.3
Zm-PepC-CBH II-ER	Submitted	Zm-*PepC*	γ-zein	CBH II	t*PepC*	SEKDEL	ER	48.4
Zm-PepC-CBH II-VSD	Submitted	Zm-*PepC*	γ-zein	CBH II	t*PepC*	DELKAEAK	Vacuole	48.6
Zm-PepC-EG-ER	Submitted	Zm-*PepC*	γ-zein	EG	t*PepC*	SEKDEL	ER	76.4
Zm-PepC-EG-CHL	Submitted	Zm-*PepC*	*C. paradoxa* FNR [7]	EG	t*PepC*	-	Chloro	75.7
Zm-PepC-EG-VSD	Submitted	Zm-*PepC*	γ-zein	EG	t*PepC*	DELKAEAK	Vacuole	76.6

[1](Christensen et al., 1992), [2](Beck et al., 1982; Bevan, 1984), [3] (Matsuoka et al., 1994),[4] (Jefferson et al., 1987), [5] (de Virgilio et al., 2008; Geli et al., 1994), [6] (Cervelli et al., 2004), [7] (Jakowitsch et al., 1996; Miles et al., 2009).

Table 2 Summary of CBH enzyme activity in green and senescent leaves of transgenic sugar cane at maturity

	Zm-*Ubi1*	Zm-*PepC*			
	CBH I-ER	**CBH I-ER**	**CBH I-VSD**	**CBH II-ER**	**CBH II-VSD**
Highest activity (green)	4.41 ± 0.29 [a]	2.38 ± 0.11	11.18 ± 1.52	5.92 ± 1.64 [b]	7.33 ± 3.78
Highest activity (senescent)	10.54 ± 2.67	5.64 ± 2.06	12.25 ± 1.12	3.26 ± 1.04	8.68 ± 0.68
Average activity top 10 events (green)	0.88 (0.11 - 4.41)	1.23 (0.58 - 2.38)	4.61 (1.21-11.18)	4.42 (2.91 - 5.92)	5.70 (5.00 - 7.33)
Average activity top 10 events (senescent)	1.60 (0.08 - 10.5)	1.79 (0.59 - 5.64)	4.46 (0.24 - 12.25)	2.42 (1.35 - 3.26)	4.95 (3.02 - 8.68)

[a] CBH I activity is presented as nmol 4-Mu min^{-1} mg^{-1} protein. [b] CBH II activity is presented as nmol reducing sugars min^{-1} mg^{-1} protein. Standard deviation is indicated for activity in the highest expressing lines. Range of activity is indicated for the average activity measured in the top ten events for each construct.

Figure Legends

Figure 1 GUS expression from the Zm-*PepC* and Zm-*Ubi1* promoters in leaves of transgenic sugar cane. The first, fully unfurled leaf from each independent transgenic event was analysed at 12 months post-transfer to soil. GUS abundance was measured using qELISA and the abundance presented is the average from two samples per plant. No GUS was detected in samples from non-transgenic sugar cane plants.

Figure 2 Accumulation of CBH I-ER in the leaves of transgenic sugar cane 12 months post-transfer to soil from the Zm-*PepC* and Zm-*Ubi1* promoter. (a) CBH I-ER activity profiles in green leaves. (b) CBH I-ER activity profiles in senescent leaves. CBH I activity was measured at pH 4.75 and 40 °C using MUL as a substrate. The average background activity, indicated on the graphs by a dashed event, from non-transgenic sugar cane was 0.04 ± 0.02 nmol 4-Mu min^{-1} mg^{-1} protein (n = 8) in extracts from green leaves and 0.01 ± 0.03 nmol 4-Mu min^{-1} mg^{-1} protein (n = 8) in extracts from senescent leaves. Average background activity plus or minus one standard deviation is indicated on the graphs by a dotted line. The average enzyme activities measured for each event (Y-axis) were normalized relative to the event with the highest activity (assigned as 100%), and graphed from lowest to highest.

Figure 3 Accumulation of CBH I and CBH II under the control of the Zm-*PepC* promoter in green and senescent leaves of transgenic sugar cane 12 months post-transfer to soil. (a) CBH I-ER and CBH I-VSD activity profiles in green leaves. (b) CBH I-ER and CBH I-VSD

activity profiles in senescent leaves. (c) CBH II-ER and CBH II-VSD activity profiles in green leaves. (d) CBH II-ER and CBH II-VSD activity profiles in senescent leaves. CBH I activity was measured at pH 4.75 and 40 °C using MUL as a substrate. The average background CBH I activity in extracts from non-transgenic sugar cane was 0.04 ± 0.02 nmol 4-Mu min^{-1} mg^{-1} protein (n = 8) for first, fully unfurled leaf and 0.01 ± 0.03 nmol 4-Mu min^{-1} mg^{-1} protein (n = 8) for senescent leaf. CBH II activity was measured at pH 4.75 and 40 °C using 1% (w/v) PASC as a substrate. The average background CBH II activity in extracts from non-transgenic sugar cane was 0.49 nmol ± 1.03 nmol reducing sugars min^{-1} mg^{-1} protein (n = 7) for first, fully unfurled green leaf and 0.14 ± 0.35 nmol reducing sugars min^{-1} mg^{-1} protein (n = 7) for senescent leaf. Background activities are indicated on the graphs by a dashed line. The background activity plus or minus one standard deviation is indicated on the graphs by a dotted event. The average enzyme activities measured for each event (Y-axis) were normalized relative to the event with the highest activity (assigned as 100%), and graphed from lowest to highest.

Figure 4 Accumulation of active EG in green leaves of mature transgenic sugar cane. (a) EG-ER, EG-CHL and EG-VSD abundance profiles. EG abundance was measured by qELISA. No EG was detected in leaf samples from non-transgenic sugar cane. The average enzyme activities measured for each event (Y-axis) were normalized relative to the event with the highest activity (assigned as 100%), and graphed from lowest to highest. (b) Correlation between EG abundance and enzyme activity in green leaves from selected events at maturity. EG abundance was measured by qELISA. Events were

selected for EG enzyme activity analysis based on EG abundance and cover the range from 90 - 425 ng EG mg^{-1} protein. At least two events per construct were selected for analysis of EG enzyme activity. EG activity was measured at pH 4.75 and 40 °C using 0.5% (w/v) CMC as a substrate. The average background EG activity in extracts from non-transgenic sugar cane was 1.49 nmol glucose ± 0.33 nmol reducing sugars min^{-1} mg^{-1} protein (n = 2). The trend line is included.

Figure 5 Western blot analysis of CBH I, CBH II and EG expression in the green leaves of transgenic sugar cane. Leaf samples from the transgenic line with the highest enzyme activity (CBH I and CBH II) or abundance (EG) were analysed by western blot. Equal amounts of protein (10 μg) were resolved by SDS-PAGE, transferred to nylon membranes and probed with affinity-purified rabbit anti-cellulase antibodies followed by the addition of HRP-conjugated to goat anti-rabbit antibody. (a) Detection of recombinant CBH I. Proteins were resolved using 10% SDS-PAGE. Purified, denatured fungal-expressed CBH I (10 ng) was included as a positive control (+). The relatively low abundance CBH I-ER protein (45 kDa) is indicated by an arrow. (b) Detection of recombinant CBH II. Proteins were resolved by 12.5% SDS-PAGE. Purified, denatured fungal-expressed CBH II (20 ng) was included as a positive control (+). (c) Detection of recombinant EG. Proteins were resolved by 7.5% SDS-PAGE. Purified, denatured bacterial-expressed EG (1 ng) was included as a positive control (+). The two relatively low abundance forms of EG-VSD (73 and 62 kDa) are indicated by arrows. Protein extracts from non-transgenic sugar cane leaves were used as negative controls on all blots (WT). Numbers on the left indicate the size of protein standard (Stds) in kDa. X-

ray films were exposed for 5 minutes and 1 minute for detection of CBHs and EG, respectively.

Figures

Figure 1

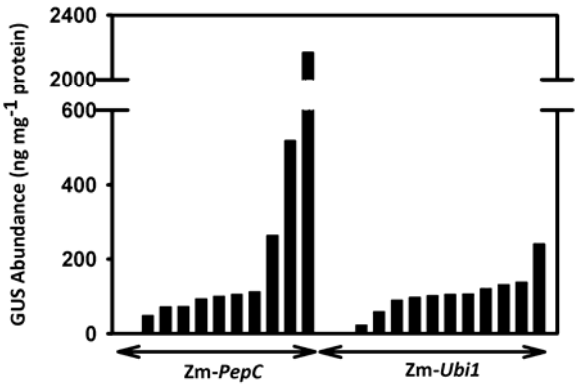


Figure 2

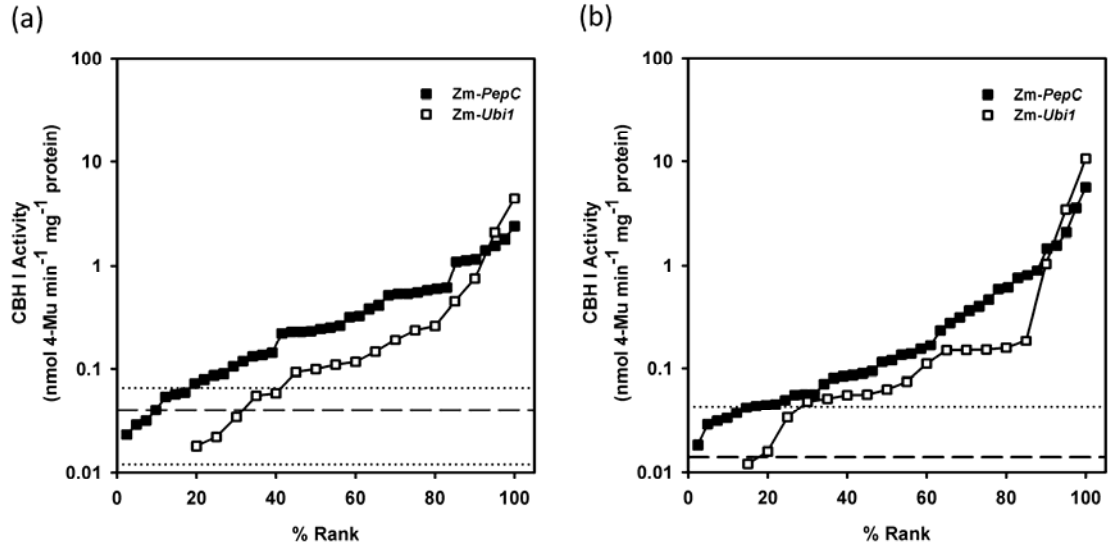

(a)
(b)
Zm-PepC
Zm-Ubi1
CBH I Activity
(nmol 4-Mu min-1 mg-1 protein)
% Rank
100
10
1
0.1
0.01
0
20
40
60
80
100

Figure 3

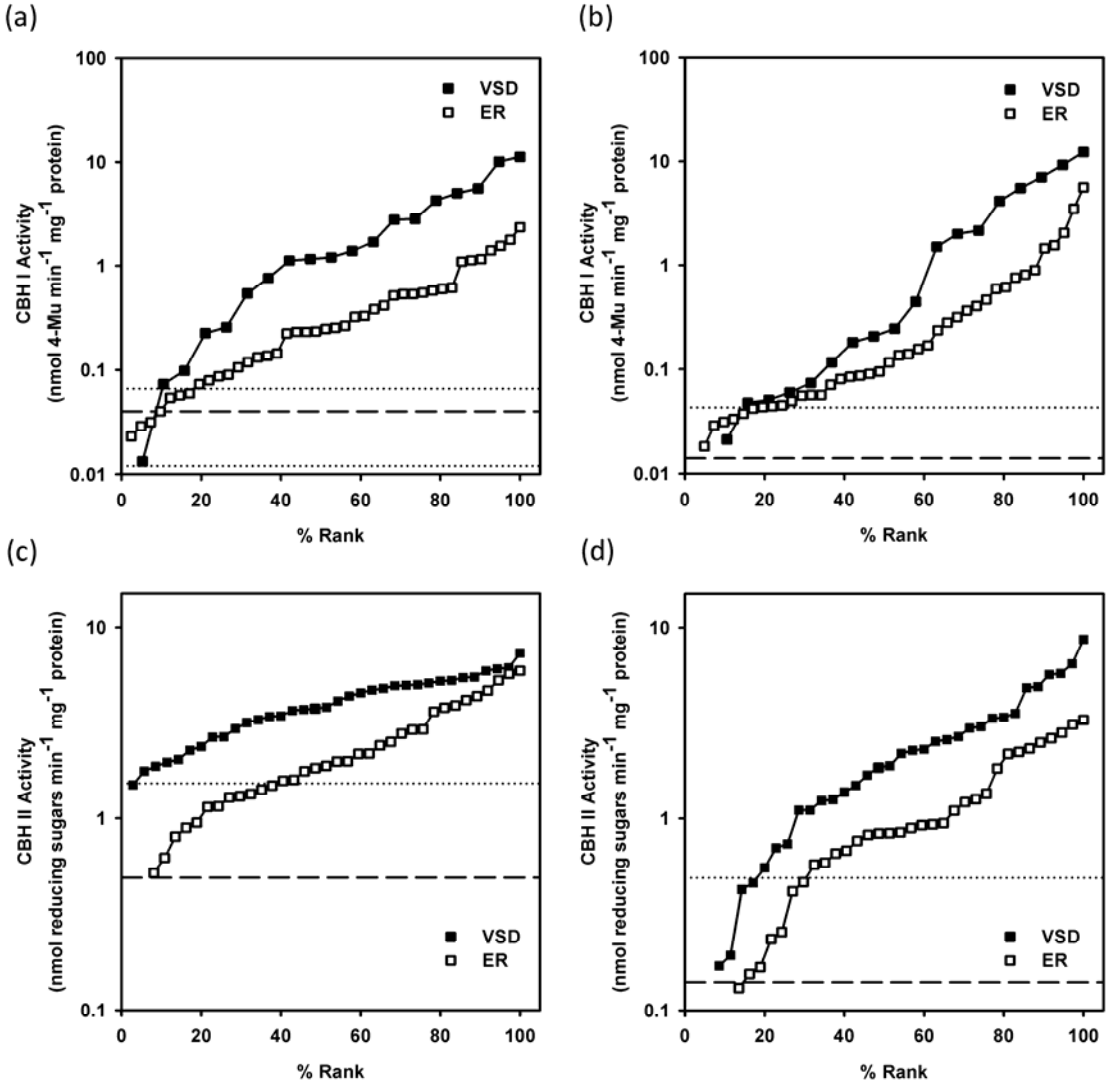
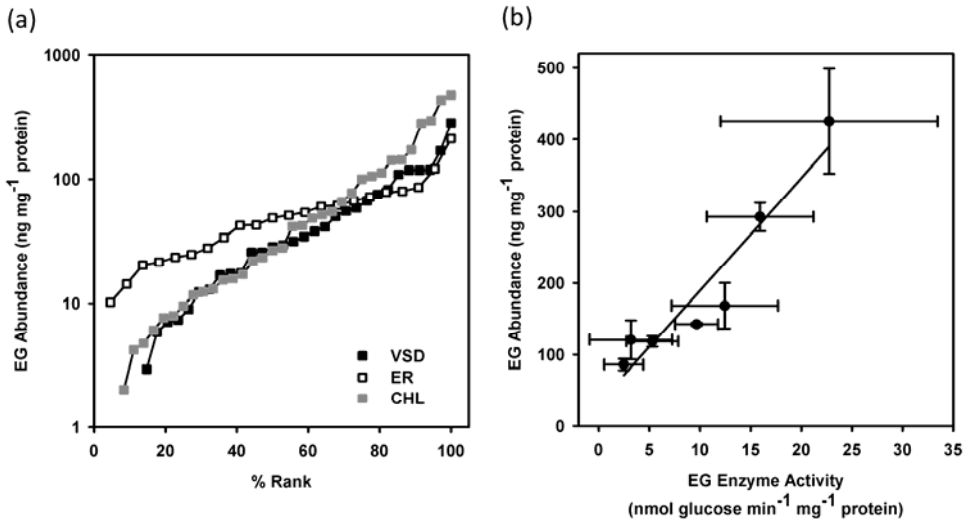

(a)
(b)
(c)
(d)
VSD
ER
CBH I Activity (nmol 4-Mu min-1 mg-1 protein)
CBH II Activity (nmol reducing sugars min-1 mg-1 protein)
% Rank

Figure 4


(a)
1000
100
10
1
EG Abundance (ng mg-1 protein)
0
20
40
60
80
100
% Rank
VSD
ER
CHL
(b)
500
400
300
200
100
0
EG Abundance (ng mg-1 protein)
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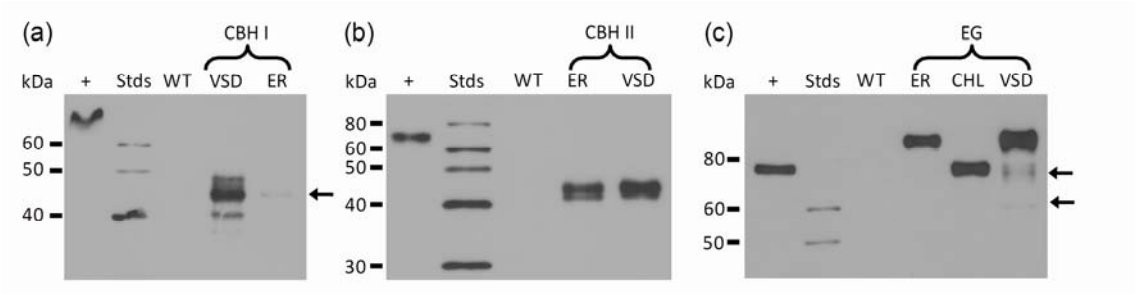
5
10
15
20
25
30
35
EG Enzyme Activity
(nmol glucose min-1 mg-1 protein)

Figure 5


(a)
CBH I
kDa
+
Stds
WT
VSD
ER
60
50
40
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CBH II
kDa
+
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VSD
80
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